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THE ROLE OF PROTEIN QUALITY CONTROL IN THE REGULATION OF E-CADHERIN AND ITS RELEVANCE IN CANCER

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ACRONYMS AND ABBREVIATIONS LIST

A

AA – amino acid

AJ – adherens junctions

ANOVA – analysis of variance

ATP – adenosine-5'-triphosphate

αctn – α-catenin

B

BSA – bovine serum albumin

βctn – β-catenin

C

CAM – chick embryo chorioallantoic membrane

CCC – cadherin-catenin complex

CCV – clathrin-coated vesicles

CFTR – cystic fibrosis transmembrane conductance regulator

CHIP – C-terminus of Hsc70-interacting protein

CHO – chinese hamster ovary

CHX – cycloheximide

CQ – chloroquine

D

DAPI – 4',6-diamidino-2-phenylindole

DGC – diffuse gastric cancer

DMSO – dimethyl sulphoxide

E

Ecad – E-cadherin

EC – extracellular cadherin

ECL – enhanced chemiluminescence

EDTA – ethylenediaminetetraacetic acid

ELISA – enzyme-linked immunosorbent assay

EMT – epithelial-mesenchymal transition

ER – endoplasmic reticulum

ERAD – endoplasmic reticulum associated degradation

ESCRT – endosomal sorting complexes required for transport

F

FBS – fetal bovine serum

G

GAPDH – glyceraldehydes-3-phosphate dehydrogenase

GC – gastric cancer

H

HDGC – hereditary diffuse gastric cancer

HRP – horseradish peroxidase

Hsc – heat shock cognate

Hsp – heat shock protein

I

IF – immunofluorescence

IGCLC – international gastric cancer linkage consortium

K

kDa – kilodalton

L

LOH – loss of heterozygosity

M

MEM – minimum essential medium

MVB – multivesicular bodies

N

NRTKs – non receptors tyrosine kinases

NSCLC – non-small cell lung carcinoma

P

PBS – phosphate-buffered saline

PM – plasma membrane

PPQC – peripheral protein quality control

PQC – protein quality control

p120ctn – p120-catenin

R

RNA – ribonucleic acid

RPMI – Roswell park memorial institute

RTKs – Receptors tyrosine kinases

S

SDS-PAGE – sodium dodecyl sulphate–polyacrylamide gel electrophoresis

shRNA – short hairpin RNA

siRNA – small interfering RNA

T

TBS – tris-buffered saline

TM – transmembrane

TPR – tetratricoeptide repeats

TSG – tumor suppressor gene

U

Ub – ubiquitin

UPS – ubiquitin-proteasome system

W

WB – western blot

WC – wound closure

WT – wild type

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ABSTRACT

E-cadherin (Ecad) is a calcium-dependent, TransMembrane (TM) glycoprotein responsible for the homophilic binding between epithelial neighboring cells. Its role in tumour development is well established, with many human carcinomas exhibiting loss of Ecad at the Plasma Membrane (PM) especially in the invasive front. Hereditary Diffuse Gastric Cancer (HDGC) is frequently associated to germline mutations of *CDH1* (gene encoding Ecad), reflecting the causative nature of Ecad loss in Gastric Cancer (GC). Our group has previously shown that Ecad expression is tightly regulated by mechanisms of Protein Quality Control (PQC), the process by which the cell examines protein folding and determines if one protein is suitable for its final destination and function. If the proper folding is not achieved, the protein is prematurely degraded. It has been shown that Ecad missense mutations associated to HDGC are recognized as unfolded and consequently eliminated by the proteasome in a process termed Endoplasmic Reticulum Associated Degradation (ERAD), but the molecular determinants of this fate are obscure. Using a Drosophila-based genetic screen, our group identified DnaJ-1 as a new Ecad interactor, suggesting that its human homolog DNAJB4 could be a molecular chaperone of Ecad.

The aims of this work are to investigate the role of the molecular chaperone DNAJB4 in the regulation of Ecad and to identify other PQC molecular components implicated in the stabilization of Ecad at the PM. For this, we used Chinese Hamster Ovary (CHO) cell lines stably expressing Wild Type (WT) Ecad or ERAD-sensitive Ecad (unfolded HDGC-associated *CDH1* missense mutant E757K), and MKN28 GC cell line expressing endogenous WT Ecad.

We demonstrate that DNAJB4 subcellular distribution is influenced by the presence of WT or unfolded HDGC-associated mutant Ecad. DNAJB4 preferencially binds to mutant Ecad and its overexpression determines the half-life of this misfolded variant, mediating its recognition for degradation in the proteasome. Interestingly, the half-life of WT Ecad is not significantly affected, sugesting that DNAJB4 is a molecular chaperone of Ecad that preferentialy regulates unfolded mutant Ecad. Posttranslational regulation of Ecad by DNAJB4 is sufficient to induce cell adhesion only in a WT Ecad cellular context, but does not reduce cell migration *in vitro*. The results of MKN28 GC cell line inoculation in a chick embryo ChorioAllantoic Membrane (CAM) showed that the anti-angiogenic and anti-invasive potential of DNAJB4 depends on Ecad expression.

We also show that the presence of unfolded Ecad at the PM leads to the recruitment of Heat shock cognate (Hsc) 70 and the Ubiquitin (Ub) ligase C-terminus of Hsc70-Interacting Protein (CHIP), indicating an involvement of the Peripheral Protein Quality

Control (PPQC) machinery, in the regulation of unfolded Ecad at the PM. Furthermore, the molecular chaperone Hsc70 regulates the endocytosis of unfolded Ecad. Interestingly, silencing of CHIP promotes the adhesion properties of Ecad, at the posttranslational level. Herein, we present evidences that molecular components of PQC mechanisms, DNAJB4, Hsc70 and CHIP, play a role in the regulation of Ecad expression and function.

RESUMO

A caderina-E é uma glicoproteína transmembranar dependente de cálcio, responsável pela ligação homofílica entre células epiteliais vizinhas. O seu papel no desenvolvimento tumoral está bem estabelecido, exibindo muitos carcinomas humanos a perda de Caderina-E na frente invasiva. O cancro gástrico difuso hereditário está frequentemente associado a mutações germinativas no *CDH1* (gene que codifica a Caderina-E), reflectindo o envolvimento da Caderina-E na patogénese do cancro gástrico. O nosso grupo mostrou anteriormente que a expressão da Caderina-E é altamente regulada por mecanismos de controlo de qualidade proteica, processo pelo qual a célula analisa o *folding* das proteínas e determina se determinada proteína é adequada para o seu destino final e função. Se o *folding* adequado não for atingido, a proteína é degradada prematuramente. Tem sido demonstrado que mutações *missense* associadas ao cancro gástrico difuso hereditário resultam predominantemente em Caderina-E *unfolded*, que é eliminada precocemente pelo proteossoma através de um processo designado por degradação associada ao retículo endoplasmático. No entanto, os determinantes moleculares deste mecanismo ainda não são conhecidos. Com base num rastreio genético realizado em *Drosophila*, o nosso grupo identificou o *chaperone* molecular Dna-J1 como um novo parceiro de interacção da Caderina-E. Esta descoberta sugere que o homólogo humano do DnaJ-1, DNAJB4, poderá ser *chaperone* da Caderina-E.

Os principais objectivos deste trabalho consistem em investigar o papel do *chaperone* molecular DNAJB4 na regulação da Caderina-E e identificar outros componentes moleculares do mecanismo de controlo de qualidade de proteinas envolvidos na regulação da estabilidade da Caderina-E na membrana plasmática. Para isso, utilizámos linhas celulares CHO (*Chinese Hamster Ovary*), que sobreexpressam de forma estável Caderina-E WT ou Caderina-E com uma mutação *missense* do *CDH1* associada ao cancro gástrico difuso hereditário (E757K), e uma linha celular derivada de cancro gástrico que expressa Caderina-E WT endógena, designada MKN28.

Neste trabalho demonstramos que a distribuição subcelular do DNAJB4 é influenciada diferencialmente pela presença de Caderina-E *wild type* ou de Caderina-E mutante, associada ao cancro gástrico difuso. Este *chaperone* molecular interage preferencialmente com a Caderina-E mutante e a sua sobreexpressão determina o tempo de meia-vida desta variante *unfolded*, mediando o seu reconhecimento para degradação no proteossoma. Curiosamente, o tempo de meia-vida da Caderina-E *wild type* não é afectado de forma significativa, sugerindo que o DNAJB4 é um *chaperone* da Caderina-E que regula preferencialmente a Caderina-E mutante. O controlo pós-traducional da

Caderina-E por DNAJB4 é suficiente para induzir adesão celular apenas no contexto de uma Caderina-E *wild type*, mas não para reduzir a migração celular *in vitro*. Os resultados da inoculação da linha celular de cancro gástrico MKN28 na membrana corioalantóide do embrião de galinha mostraram que o potencial anti-angiogénico e anti-invasivo do DNAJB4 depende da expressão da Caderina-E.

Neste trabalho mostramos também que a presença de Caderina-E *unfolded* na membrana plasmática conduz ao recrutamento de Hsc70 e da ligase de ubiquitina CHIP. Esta observação indica o envolvimento da maquinaria de controlo de qualidade periférico na regulação da Caderina-E *unfolded* na membrana. Além disso, o *chaperone* molecular Hsc70 regula a endocitose da Caderina-E *unfolded*. Uma outra observação interessante é que o silenciamento de CHIP promove, ao nível pós-traducional, as propriedades de adesão da Caderina-E.

Em conclusão, neste trabalho apresentamos evidências de que os componentes moleculares dos mecanismos de controlo de qualidade, DNAJB4, Hsc70 e CHIP desempenham um papel na regulação da expressão e função da Caderina-E.

INTRODUCTION.01

E-CADHERIN STRUCTURE AND FUNCTION. 1.1

E-cadherin (Ecad) is a TransMembrane (TM) glycoprotein with 120 kiloDalton (kDa), localized on the basolateral surface of epithelial cells in regions of intercellular contact forming Adherens Junctions (AJ) [1-3]. These dynamic structures contribute to the physical binding of cells by the establishment of homophilic interactions between two Ecad molecules of adjacent cells [4-6]. The regulation of cell-cell contacts by AJ is essential during embryonic morphogenesis, and in maintaining architecture and homeostasis in adult tissues [7].

The mature Ecad is organized in three major structural domains: a large extracellular domain of about 550 Amino Acid (AA) residues, a single TM domain, and a short cytoplasmic domain of about 150 AA (Figure 1) [1,2,8,].

The ectodomain comprises five Extracellular Cadherin (EC) repeat domains that are bridged by calcium-ions (Figure 1) [1,8]. The interaction between calcium-ions and ECs is crucial for the stable conformation (rod-like structure) of Ecad, for protection against proteases, and for cell-cell adhesion [9].

Ecad highly conserved cytoplasmic domain interacts with β-catenin (βctn) at the C terminus of the protein and with p120-catenin (p120ctn) at the justamembrane domain (Figure 1). βctn binds directly to α-catenin (αctn), and the Cadherin-Catenin Complex (CCC) links to the actin filaments (Figure 1) and several actin-binding proteins [1,7,10-16]. p120ctn is important to stabilizes Ecad at the Plasma Membrane (PM) [1,17-20].

Ecad is involved in many biological processes including morphogenesis, cell adhesion, cytoskeletal organization, signalling, and cell sorting/migration [21].

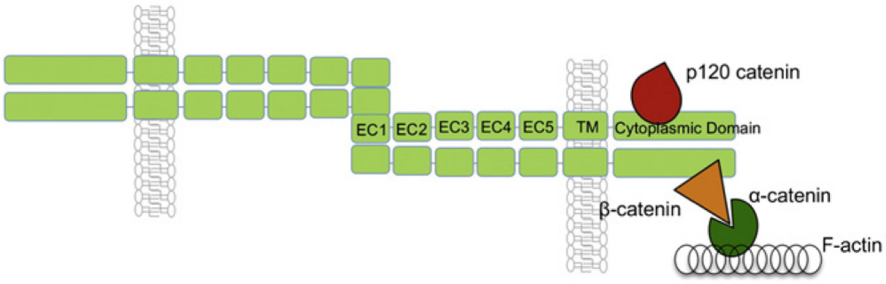

Figure 1. Schematic overview of the classical cadherin-catenin complex. E-cadherins are composed by an extracellular domain, a TM domain and a cytoplasmic domain, which interacts with actin filaments via catenins (Adapted from Paredes *et al*. *Biochim Biophys Acta*, 2012).

POSTTRANSLATIONAL REGULATION OF E-CADHERIN. 1.2

Intracellular trafficking and posttranslational modifications are important regulators of the levels of Ecad at the cell surface.

An important level of posttranslational regulation of Ead is by glycosylation. N-glycosylation of extracellular domain of Ecad seems to be essential for its expression, folding and trafficking [7].

Ecad is synthesized as a 135 kDa precursor form that undergoes cleavage by proprotein convertase family of proteins associated with the trans-Golgi network that remove the N-terminal pro-region [22,23]. This processing is essential for correct folding of the extracellular portion of the mature Ecad [23,24].

After this cleavage Ecad follows the secretory pathway from the trans-Golgi network to the PM. During this process, βctn binds to Ecad and these two proteins are transported to the PM as a complex. At the cell surface, assembly into functional CCC occurs. Binding of p120ctn to the Ecad justamembrane domain stabilizes and prevents entry of Ecad into degradative endocytic membrane trafficking pathway, by hampering the binding of adaptor complexes that would recruit the clathrin-coated pits [25,26].

Several different internalization pathways, including clathrin-dependent and clathrin-independent mechanisms, mediate Ecad endocytosis. It seems likely that the internalization machinery utilized by Ecad is highly cell or tissue type dependent [26].

Phosphorylation and ubiquitination of Ecad are additional forms of posttranslational regulation [7]. Activation of Receptors Tyrosine Kinases (RTKs) and Non Receptors Tyrosine Kinases (NRTKs) promotes the phosphorylation of βctn and the intracytoplasmic tail of Ecad (Figure 2) [25]. This posttranslational modification induces the dissociation of p120ctn from Ecad and the recruitment of Hakai, a c-Cbl-like E3 ubiquitin (Ub) ligase (Figure 2) [25,27]. Binding of Hakai leads the loss of Ecad interaction with the actin cytoskeleton and may initiate the activation of intracellular signalling pathways (Figure 2) [25]. The E3-ligase function of Hakai mediates the transfer of Ub chains to Ecad and βctn through the E1-E2 ubiquitination system (see The Ubiquitin-Proteasome System section) (Figure 2). Ubiquitinated βctn is degraded in the proteasome and ubiquitinated Ecad-Hakai complex is eliminated by the lysosome or else Ecad is recycled back to the PM (Figure 2) [25,27].

Ecad trafficking deregulation is associated to epithelial cancer progression [7].

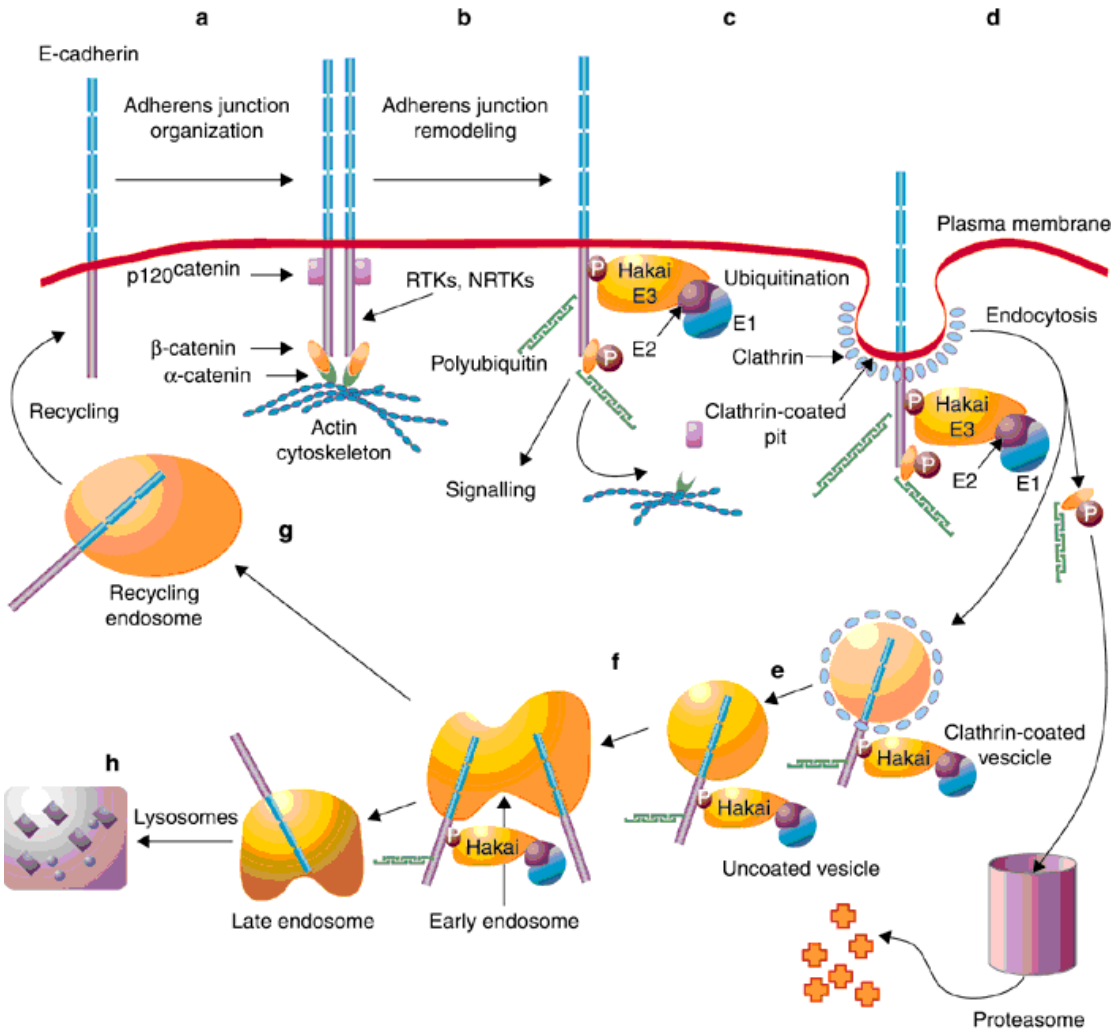

Figure 2. A model for Hakai in the dynamic regulation of Ecad. a - At steady-state, Ecad are organized in multiprotein complexes in AJ. b - Activation of RTKs and NRTKs promotes the phosphorylation (P) of βctn and the intracytoplasmic tail of Ecad, the recruitment of Hakai in a tyrosine-phosphoylation dependent manner, the loss of Ecad interaction with the actin cytoskeleton, and the disruption of AJ. c - Ecad bound to Hakai may initiate the activation of intracellular signalling pathways, whereas the E3-ligase function of Hakai mediates the transfer of Ub chains to Ecad and βctn through the E1–E2 ubiquitination system. d - Ubiquitinated βctn is degraded in the proteasome, but ubiquitinated Ecad-Hakai complexes are likely internalized by clathrin-coated pits, e - which are then rapidly uncoated and fuse to early endosomes. f - In endosomes, depending on a balance between ubiquitination and de-ubiquitination systems, g - Ecad may be either recycled back to the cell surface, h- or targeted for degradation in the lysosomal compartment (Adapted from Pece S and Gutkind JS, *Nat Cell Biol,* 2002).

Ecad gene *(CDH1*, located at human chromosome 16q22.1) is considered a Tumor Suppressor Gene (TSG) wich plays an important role as suppressor of invasiveness and epithelial cell migration [28,29].

Genetic or epigenetic alterations in this gene often result in abnormal Ecad expression or function. Loss of Ecad is a defining characteristic of Epithelial-Mesenchymal Transition (EMT), a process associated with invasion and metastasation that are a hallmark in late carcinogenesis (Figure 3) [30-32].

In sporadic lobular breast cancer and Diffuse Gastric Cancer (DGC), Ecad inactivation is associated with somatic mutations of *CDH1*, as well as loss of heterozygosity (LOH), promoter hypermethylation, aberrant glycosylation, or overexpression of transcriptional repressors (*e.g.* Snail, Slug, Sip5 and Ets) [33-35].

In 1998, Guilford *et al.* identified in three Maori families the first germline mutations in Ecad gene associated with Hereditary Diffuse Gastric Cancer (HDGC) [36].

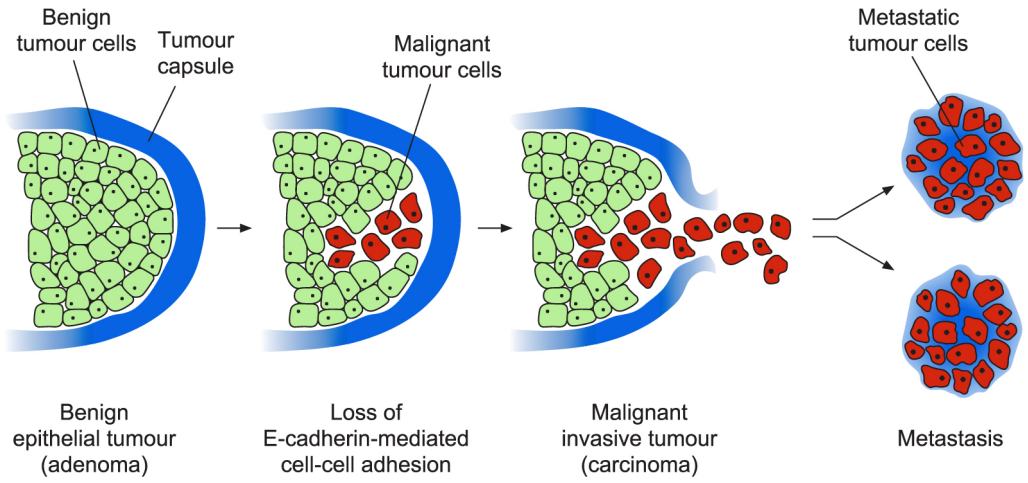

Figure 3. EMT in cancer progression and metastasis. Loss of Ecad-mediated cell adhesion contributes to the transition from benign, non-invasive tumours (adenoma) to malignant, invasive tumours (carcinoma) (Adapted from Christofori G and Semb H, *Trends Biochem Sci*, 1999).

1.3.1. Hereditary Diffuse Gastric Cancer

HDGC is a rare (30% of the familial cases, which represent 1-3% of all Gastric Cancer (GC) cases) autosomal dominant cancer susceptibility syndrome. Germline mutations of

CDH1 are the only known genetic cause of HDGG [37-38].

Stringent criteria for genetic test were defined by the International Gastric Cancer Linkage Consortium (IGCLC) in 1999, and include the following: (i) two or more documented cases of GC in first/second degree relatives, with at least one DGC diagnosed before 50 years of age; (ii) three or more cases of documented GC in first/second degree relatives, with at least one DGC diagnosed at any age; (iii) single individual with DGC diagnosed before 40 years age without a family history; or (iv) single individual and families with diagnoses of both DGC (including one case below the age of 50 years) and lobular breast cancer [36,37].

The average age of onset of HDGC is 38 years, with a range of 14-69 years [37,38]. The estimated cumulative risk of GC by age 80 years is 63%-83% for women and 40%-67% for men [38]. Women also have a 39%-52% risk for developing lobular breast adenocarcinoma [36].

Prophylactic total gastrectomy is the only preventive treatment and is recommended to the *CDH1* mutation carriers after 20 years of age, especially if they are carriers of nonsense mutations [37-39]. Missense mutations account for approximately 25% of all HDGC cases harboring *CDH1* mutations [40].

Histologically, HDGC is characterized by an infiltration of isolated signet-ring cells into the gastric wall (Figure 4), causing thickening of the wall (*linitis plastica*) without forming a distinct mass [39,41].

HDGC-associated missense mutations can lead to an unfolded Ecad protein that is surveyed by Protein Quality Control (PQC) mechanisms and prematurely degraded in the proteasome [42,43].

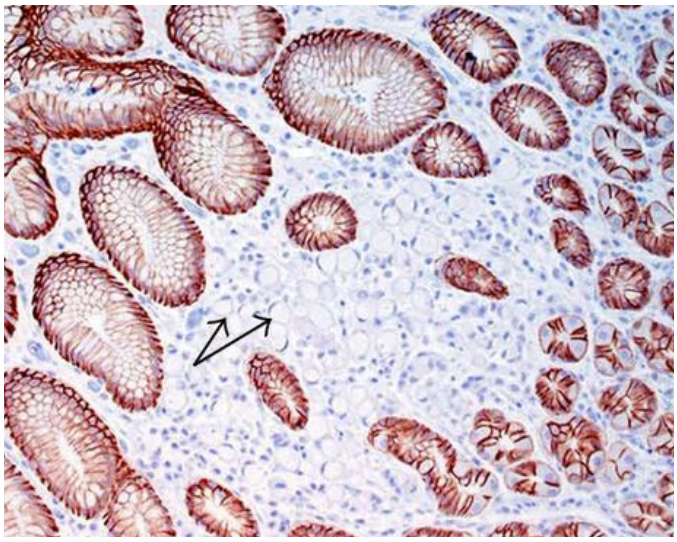

Figure 4. Typical invasive focus of intramucosal signet-ring cell (diffuse) adenocarcinoma with immunostaining for Ecad. Tumor cells (arrows) show decreased Ecad expression, the normal background epithelium serves as an internal control (Adapted from Wilcok R *et al*, *Patholog Res Int*, 2011).

PROTEOSTASIS AND PROTEIN QUALITY CONTROL. 1.4

Eukaryotic protein homeostasis, or proteostasis, enables healthy cell and organismal development as well as ensures a better adaptation to aging. Proteostasis is a complex and integrated biological network within cells, comprising various pathways that include the biogenesis, folding, trafficking and turnover of proteins [40]. The accumulation of misfolded, aggregation prone and potentially cytotoxic proteins can be generated by mutations, transcriptional and translational errors or cellular and environmental stresses. To advert these dangers for protein homeostasis, cells have developed powerful strategies of PQC [44,45].

PQC is a general term used to refer the mechanisms by which cells control and survey proteins folding and decides if the protein is suitable for its final destination and function [46,47]. Deficiencies in PQC system lead to many metabolic, oncological, neurodegenerative, and cardiovascular disorders [40].

PQC consists of a large arsenal of molecular chaperones and proteolytic systems.

Protein folding, unfolding, and refolding are constantly occurring throughout the lifetime of nearly all proteins [44]. Molecular chaperones promote folding and maintenance of conformation within the cell largely by minimizing misfolding and aggregation, but the chaperones also escort terminally misfolded proteins or irreversibly damaged proteins to the proteolytic pathways for degradation [48].

The main proteolytic systems associated to the PQC are Ubiquitin-Proteasome System (UPS) and the autophagy pathway.

1.4.1. The Ubiquitin-Proteasome System

The UPS is the main proteolytic system involved in the selective degradation of soluble proteins. This pathway is implicated in numerous cellular events where protein degradation is required either to dispose of obsolete proteins or to regulate various biological processes. Degradation of a protein by the UPS involves two different successive steps: the first step implies the covalent attachment of small (8.5 kDa) regulatory polipeptides called Ub to the target protein, in a process known as ubiquitination; the second step involves recognition and final degradation of the targeted protein by the proteasome (Figure 5) with the release of free and reusable Ub [49].

Ubiquitination is canonically achieved via an enzymatic cascade. In the first step of this cascade, an Ub molecule is linked by its C-terminal AA to a cysteine residue of E1 Ub-activating enzyme, in an adenosine-5'-triphosphate (ATP) - consuming process (Figure 5). The activated Ub moiety is then transferred to the active site of E2 (Ub-carrier protein or Ub-conjugating enzyme) (Figure 5). In the last step, the Ub is transferred from the E2 to the target protein with the help of an E3 Ub-protein ligase enzyme that specifically binds to the substrate (Figure 5) [50].

The human genome encodes 2 Ub-specific E1 activation enzymes, about 30 E2 conjugation enzymes, and more than 1000 E3 ligases providing a great versatility in substrate recognition and enabling diversity in Ub chain linkages added to substrates [49,51,52]. Most proteosomal substrates have to be polyubiquitinated in order to be properly recognized by the proteosome (Figure 5) The prime tag for proteasomal degradation is a chain of four or more Ub moieties covalently linked to a lysine residue of the substract. Ub has seven internal lysines (K6, K11, K27, K29, K33, K48 and K63) that can bind to other Ub monomers to form polyubiquitin chains [53]. K48-linked polyubiquitin chains represent the canonical proteasomal degradation tag, but recently other chains were also identified as being able to target substrates for degradation in the proteosome [54].

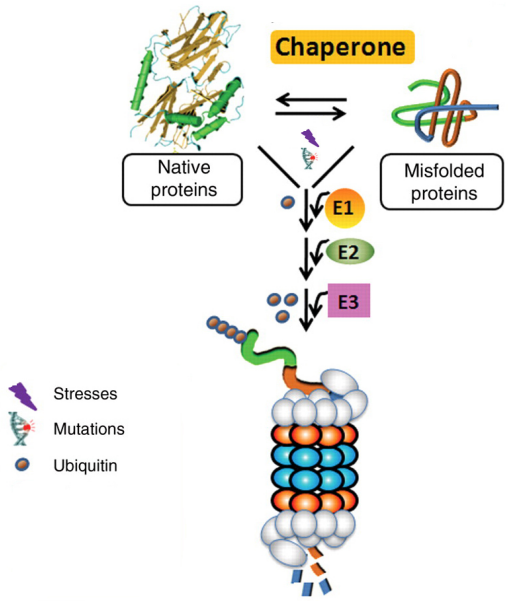

Figure 5. An illustration of UPS in the cell. Chaperones facilitate the folding of nascent polypeptides and refolding of misfolded proteins, prevent the misfolded proteins from aggregating, directing terminally for degradation by the UPS. The UPS degrades both misfolded/damaged proteins and most unneeded native proteins in the cell. This process involves two steps: first, covalent attachment of Ub to a target protein by a cascade of chemical reactions catalysed by the Ub-activating enzyme (E1), Ub-conjugating enzymes (E2), and Ub ligase (E3); and then the degradation of the target protein by the proteasome. (Adapted from Wang X *et al, Circ Res,* 2013).

1.4.2. Autophagy

Autophagy was initially thought to be a form of cell response and adaptation to lack of nutrients, it is now realized that autophagy is a highly regulated and multipurpose system. The best-characterized signal for the activation of autophagy is nutrient deprivation. When nutrients are insufficient, this pathway allows a cell to break down its own components, including proteins and organelles and recycle important molecules. It is a very important PQC system that represents the adaptation of the cell to starvation/nutrient deprivation allowing the cell to survive until there is food available in the medium [55,56].

To date, at least three types of autophagic pathways have been described, which differ in their routes to lysosomes: macroautophagy (also commonly called “autophagy”), microautophagy and Chaperone-Mediated Autophagy (CMA). The essential component of these proteolic systems is the lysosome, a single membrane vesicle that contains in its lumen a large variety of cellular hydrolases including proteases, lipases, glycosidades, and nucleotidases [54].

ENDOPLASMIC RETICULUM QUALITY CONTROL. 1.5

To accomplish Endoplasmic Reticulum (ER) quality control, cells have a complex network of molecular chaperones associated with the ER, which interact with nascent proteins in the ER lumen, promoting the folding of client proteins until a proper native state is achieved. When native structure is accomplished the client proteins leave the ER and are transported to the Golgi apparatus where they are modified, sorted and sent towards their final destinations [45,47]. Chemical chaperones are organic molecules that seem to facilitate this process in the ER, promoting folding and assembly of client proteins (Figure 6). If the proper folding is not achieved, namely due to the presence of a missense mutation, the misfolded protein is retrotranslocated into the cytoplasm to be eliminated by the proteasome in a process termed Endoplasmic Reticulum Associated Degradation (ERAD) [57].

Simões-Correia *et al.* showed that Ecad expression is tightly regulated by PQC *in vitro*, and Ecad mutations affecting the justamembrane domain lead to recognition as misfolded in the ER and consequent premature degradation by the proteasome. Moreover, they showed that chemical chaperone dimethyl sulphoxide (DMSO) could rescue Ecad with HDGC-associated mutations from proteosome-dependent degradation, thus favoring the accumulation of Ecad at the PM [42].

1.5.1. Chaperones Associated to the Endoplasmic Reticulum Quality Control

The main chaperones associated with the ER are calnexin, calreticulin, Grp78 (also known BiP) and ERp57 [52].

However, it has recently been proposed that DNAJ proteins can be associated to the ER and act in misfolding recognition of TM proteins [58]. DNAJ proteins belong to the Heat shock protein (Hsp) 40 family and have been divided into three classes I, II and III, also known as class A, B and C, respectively [59]. Humans have 41 different DNAJ-encoding genes [60].

DNAJB1 (classical Hsp40) interacts with Hsp70 through its J domain and plays a role in regulation of ATPase activity of Hsp70, and in binding of unfolded proteins to Hsp70 [59-61].

DNAJB4 (DNAJ homolog, subfamily B, member 4) have a high homology with DNAJB1 and is described to be a molecular chaperone with essential role in protein stabilization

[62]. It has been demonstrated that DNAJB4 has little impact in refolding and aggregation suppression [63], but its binding to unfolded substrates has been described [61,62]. DNAJB4 acts as a tumor supressor in Non-Small Cell Lung Carcinoma (NSCLC) model, while inhibiting tumorigenesis and metastasis [64]. There are no reports exploring the potential of DNAJB4 as a molecular chaperone of Ecad, or demonstrating its relevance for GC progression.

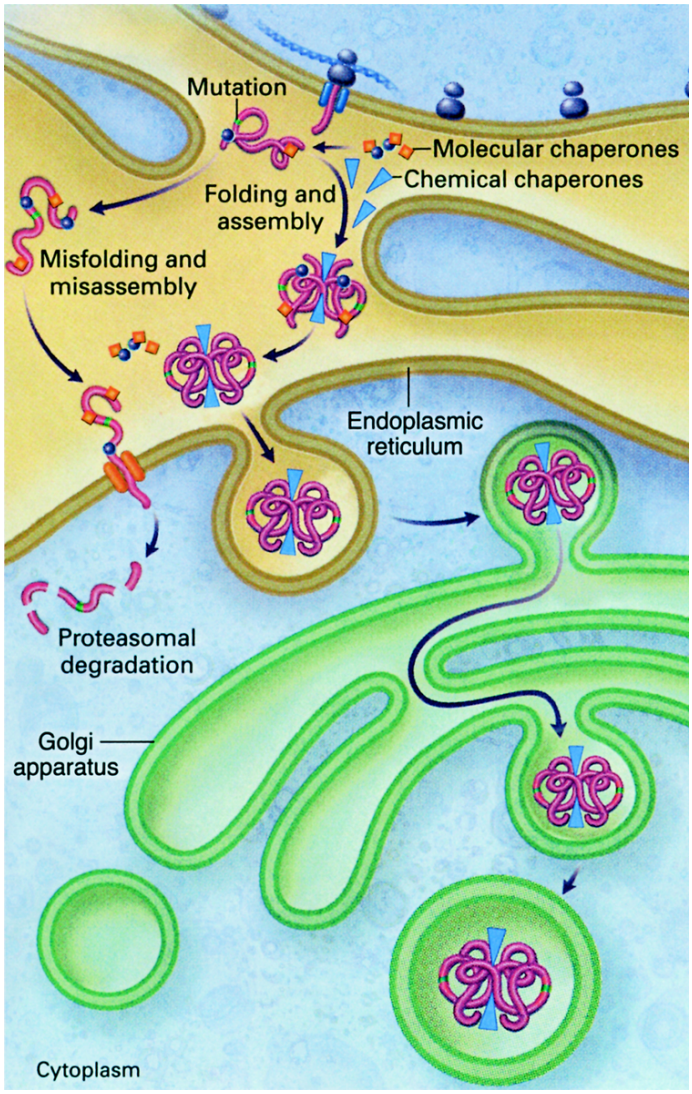

Figure 6. Putative mechanism of chemical chaperones in the ER and Golgi apparatus. Newly synthesized polypeptides are translocated into the lumen of the ER. Folding is facilitated by interaction with molecular chaperones. If the polypeptide is mutated, there is misfolding and misassembly and then dislocation into the cytoplasm for proteosomal degradation after dissociation of the molecular chaperones. In the presence of chemical chaperones, folding and assembly of the mutated polypeptide is presumably facilitated so that it can exit the ER by vesicular transport to the Golgi and then from Golgi to PM and/or extracellular fluid. Molecular chaperones dissociate within the ER but chemical chaperones are associated throughout the secretory pathway because they presumably saturate all of the transport compartments. (Adapted from Perlmutter DH, *Pediatric Research*, 2002).

Peripheral Protein Quality Control (PPQC) has recently been proposed to be a specialized pathway for the regulation of unfolded proteins at the PM. In this pathway, unfolded substrate is internalized and subsequently degraded in the lysosomal compartment (Figure 7). Lysosome-dependent degradation depends of the Endosomal Sorting Complexes Required for Transport (ESCRT) to recognize ubiquitinated proteins in the endosome and it promotes cargo delivery into multivesicular bodies (MVB)/lysosome and protein degradation (Figure 7) [65,66].

The molecular components of PPQC seem to be partially shared with ER quality control [67]. Heat shock cognate (Hsc) 70 has their main chaperoning activity within the cytoplasm [68,69]. However, it has been reported recently, that Hsc70/Hsp90 and Hsc70/C-terminus of Hsc70-Interacting Protein (CHIP) are part of the PPQC machinery, and that they are involved on the recognition and ubiquitination of unfolded proteins at the cell surface (Figure 7) [65].

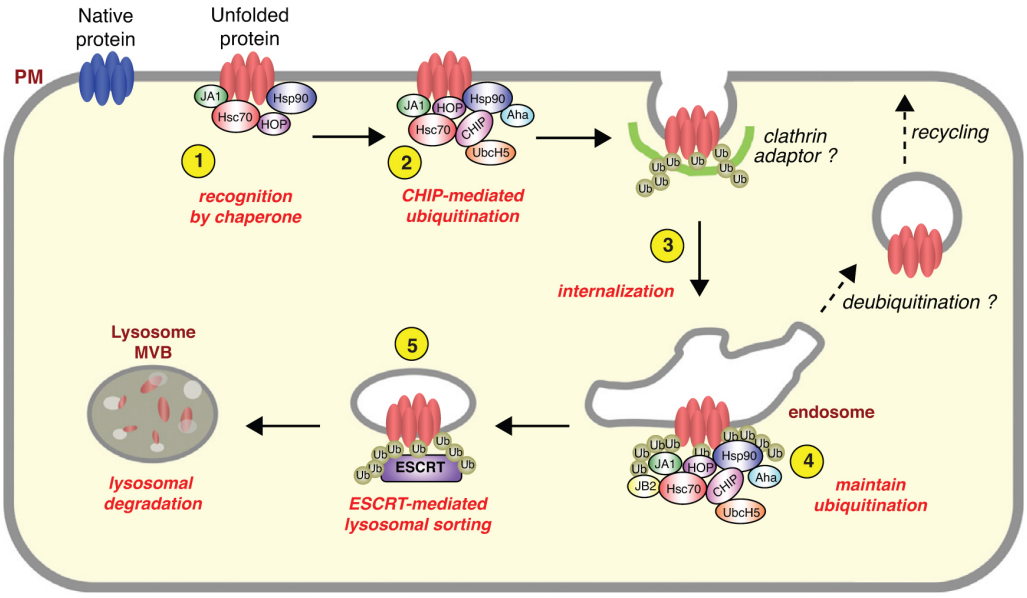

Figure 7. Working model for the PPQC network. 1- Unfolded PM protein is recognized by Hsc70/DNAJA1, possibly in conjunction with Hsp90/Hop/Aha1. 2- Recruitment of CHIP/UbcH5 leads to ubiquitination of the unfolded substract. 3- Recruitment of endocytic adaptors to endocytose unfolded substrate. 4, 5- Depending on the folding propensy of the cargo and the proteostasis network state, interaction with chaperones and co-chaperones may favor the client refolding, deubiquitination and recycling to the PM. Irreversible unfolded PM substrates will lead to persistent ubiquitination (involving CHIP and/or other E3 ligases), recruitment of ESCRT components and sorting to the lysosome compartment for degradation (Adapted from Okiyoneda T *et al*, *Curr Opin Cell Biol*, 2011).

CHIP is an E3 Ub ligase containing three TetratricoPeptide Repeats (TPR) domains at its N-terminal and an U-box domain at its C-terminal and plays a central role in protein triage decision [70,71].

Previous studies show that CHIP complex recognizes the mutant variant ΔF508 of Cystic Fibrosis Transmembrane Conductance (CFTR). This complex catalyzes its ubiquitination that diverts unfolded proteins into the lysosomal degradative pathway [66].

This quality control mechanism is thought to be determinant for the clearance of unfolded proteins at the PM that either result from genetic mutations or denaturing extracellular stimuli [67].

AIMS.02

AIMS.02

The main objective of this project is to understand the importance of PQC in the regulation of Ecad and evaluate its significance in cancer. To this end the following specific objectives were sought:

- Determine the subcellular distribution of DNAJB4 in the context of WT or mutant Ecad;
- Explore *in vitro* the role of DNAJB4 in the stabilization and/or degradation of Ecad;
- Understand if DNAJB4 has an impact in cellular adhesion and migration;
- Investigate *in vivo* the role of DNAJB4 in invasion and angiogenesis;
- Analyse the stability of WT Ecad at the PM after extracellular calcium depletion;
- Analyse the stability of Ecad mutant at the PM after folding/unfolding stimulus with DMSO;
- Test if PPQC components, namely Hsc70 and CHIP, are recruited by unfolded Ecad to the PM;
- Determine if the PPQC machinery, namely Hsc70 and CHIP, regulates cell adhesion.

MATERIAL AND METHODS.03

3.1 CELL CULTURE, TRANSFECTIONS AND TREATMENTS

Chinese Hamster Ovary (CHO) cells (CCL-61; ATCC™, Barcelona, Spain) were grown in MEM Alpha (Gibco®, Life Technologies™, Barcelona, Spain) and MKN28 GC cell line was maintained in RPMI medium (Gibco®), both supplemented with 10% Fetal Bovine Serum (FBS; HyClone®, Salt Lake City, Utah) and 1% penicillin-streptomycin (Gibco®). All cell lines were maintained in a humidified incubator with 5% CO_2 at 37°C.

CHO stable cell lines, Wild Type (WT) human Ecad, mutant Ecad (E757K) or empty vector (Mock), were established previously [42] and maintained in the presence of antibiotic selection with 5μg/mL blasticidin (Gibco®).

For stable silencing of Ecad, MNK28 were transduced by lentiviral infection of a short hairpin RNA (shRNA) and corresponding control (Table 1, annexes), using polybrene. Stable cell lines were established by antibiotic selection with 5μg/mL puromycin (Sigma-Aldrich®, Sintra, Portugal).

For transient transfections, $2x10^5$ cells were seeded in 6-well plates and, at 30% confluence, they were transfected with 1μg of vector DNA (Table 1, annexes) or 50nM of small interfering RNA (siRNA) (Table 2, annexes), using Lipofectamine® 2000 Transfection Reagent (Invitrogen™, Life Technologies™) according to the manufacture procedure.

For the protein synthesis inhibition, 24h after transfection, CHO cells were treated with 50μM of Cycloheximide (CHX; Sigma-Aldrich®) for 0, 2, 4 and 8h.

For proteasome inhibition, transiently transfected cells were incubated for 8h with 10μM of MG132 (CalBioChem®, Millipore™, Billerica, Massachusetts, USA). Lysosomal inhibition was obtained with Chloroquine (CQ; Sigma-Aldrich®), according to times and concentrations indicated. The chemical chaperone 2% DMSO (Sigma-Aldrich®) effect was induced for 24h.

To destabilize WT Ecad at the PM, 48h after transfection, MKN28 and CHO cells were incubated for indicated times in medium without calcium supplemented with 2mM ethylenediaminetetraacetic acid (EDTA; Stratagene®, La Jolla, California, USA). To stabilize mutant Ecad at the PM, transiently transfected cells were treated with 2% DMSO for 24h. Afterwards, destabilization was recovered by removing the folding stimulus (incubation with normal medium for 4h).

For inhibition of endocytosis, CHO cells were incubated for 15min with 0,4M sucrose.

PROTEIN EXTRACTION, QUANTIFICATION AND WESTERN BLOT .3.2

Cells lysates were obtained with cold Catenin lysis buffer[1] enriched with a protease inhibitor cocktail (Roche, Amadora, Portugal) and phosphatase inhibitor cocktail (Sigma-Aldrich®). To separate the proteins from cellular debris, cells lysates were spun down and the supernatant (proteins) were quantified using a modified Bradford assay (Bio-Rad, Amadora, Portugal). 40μg of total protein was denatured in loading buffer[2], separated in 7.5% Sodium Dodecyl Sulphate–PolyAcrylamide Gel Electrophoresis (SDS-PAGE), and electroblotted to nitrocellulose membrane (Bio-Rad). Membranes were blocked overnight at 4°C with 5% non-fat dry milk (Molico®, Nestlé®, Vevey, Switzerland) and 0.5% Tween-20 in Tris-Buffered Saline (TBS), and immunoblotted with primary antibodies (Table 3, annexes). HorseRadish Peroxidase (HRP) - conjugated secondary antibodies (Table 4, annexes) were used accordingly, followed by Enhanced ChemiLuminescence (ECL) detection (Bio-Rad). Immunoblots were quantified with Quantity One® Software (Bio-Rad).

IMMUNOPRECIPITATION AND CELL SURFACE BIOTINYLATION .3.3

To immunoprecipite Ecad, 400μg of protein were coupled to a mouse monoclonal anti-Ecad antibody (BD Transduction Laboratories™, BD Biosciences, San Jose, California, USA), and immunocomplexes were incubated with PureProteome™ Protein G Magnetic Beads (Millipore™), washed and eluted in loading buffer, according to manufacture instructions.

Cell surface biotinylation was performed at 4°C by incubation with freshly prepared EZ-Link®Sulpho-NHS-SS Biotin (Thermo Scientific™, Pierce Biotechnology, Rockford, Illinois, USA) at 0,5mg/mL in phosphate-buffered saline (PBS) - PLUS[3] for 30min. The reaction was quenched with PBS-PLUS containing 100mM glycine (NZYTech, Lisboa, Portugal). Whole cell lysates were prepared according to the protocol described above. An aliquot of 400 μg of total protein was incubated on a rotator overnight with 50μL of Streptavidin Sepharose Beads (GE Healthcare, Little Chalfont, UK) at 4°C. To separate

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[1] 1% Triton X-100, 1% Nonidet P-40 in PBS

[2] 62.5mM Tris–HCl (pH 6.8), 2% SDS, 10% glycerol, 0.01%, bromophenol blue, and 5% β-mercaptoethanol

[3] PBS supplemented with 1mM $CaCl_2$ and 1mM $MgCl_2$

the membrane fraction from the cytoplasmic fraction the samples were spun down, and the pellet (membrane fraction) was washed, eluted in loading buffer and analysed by WB.

SUBCELLULAR PROTEIN FRACTIONATION.3.4

For subcellular fraccionation, $5x10^5$ CHO cells (stably expressing WT or unfolded E757K mutant Ecad) were harvested and fractionated into different subcellular extracts using the subcellular protein fractionation kit (Thermo Scientific™, Pierce Biotechnology) according to manufacture protocol. 15% of the total content from the membranous and cytoplasmic fractions was analysed by WB with the primary antibodies against Ecad, V5-tag and Calnexin (Table 3, annexes).

SLOW AGGREGATION ASSAY.3.5

Wells of 96-well plate were coated with 50μL of agar solution[4]. Transiently transfected CHO cells were detached with 0.05% trypsin-EDTA (Gibco®) and ressuspended in culture medium. A suspension of $1x10^5$ cells/mL was prepared and $2x10^4$ cells were seeded in each well. The plate was incubated at 37°C in a humidified chamber with 5% CO_2. Aggregation was evaluated in an inverted fluorescent microscope (Leica DMIRE2, x10 magnification) 48h after seeding, and images captured using a digital camera (Leica DFC 350 FX).

CELL MIGRATION ASSAY.3.6

The migratory/motility behavior of CHO cells stably expressing WT Ecad (in control conditions or upon DNAJB4 transient transfection) was analysed in an *in vitro* wound healing assay. Cells were grown to confluence in 6-well plates, an artificial wound was created with a yellow pipette tip, and cells were carefully washed twice with PBS to remove detached cells. Migration was assessed by measuring the distance between wound edges at time intervals (0 and 4h). The cells were visualized by phase-contrast

[4] 100mg Bacto-Agar in 15mL of sterile PBS

light microscopy at x5 magnification and images captured using a Leica DFC 350 FX digital camera mounted on a Leica DMRIE2 microscope.

3.7 CAM ASSAY, IMMUNOHISTOCHEMISTRY AND STATISTICAL ANALYSIS

The chick embryo ChorioAllantoic Membrane (CAM) assay was used to evaluate the angiogenic response and invasive potential of MKN28 GC cells stably transduced as described above and transiently transfected with DNAJB4. Fertilized chick (*Gallus gallus*) eggs obtained from commercial sources were incubated horizontally at 37.8°C in a humidified atmosphere and referred to embryonic day (E). On E3 a square window was opened in the shell after removal of 1.5-2mL of albumin to allow detachment of the developing CAM. The window was sealed with a transparent adhesive tape and the eggs returned to the incubator. $1x10^6$ cells re-suspended in 10µL of complete medium, were placed on top of E10 growing CAM into a 3mm silicon ring under sterile conditions. The eggs were re-sealed and returned to the incubator for an additional 3 days. The embryos were euthanized by adding 2mL of fixative in the top of the CAM. After removing the ring, the CAM was excised from the embryos and photographed *ex ovo* under a stereoscope at x20 magnification (Olympus, SZX16 coupled with a DP71 camera). The number of new vessels (less than 15µm diameter) growing radial towards the ring area was counted in a blind fashion manner. The CAM assay was performed by Marta Teixeira Pinto of IPATIMUP.

Excided CAM were fixed in 10% neutral-buffered formalin, paraffin-embedded for slide sections and stained with hematoxilin-eosin for histological examination. Tumour sections obtained from CAM were de-paraffinized, re-hydrated with graded ethanol and washed in destiled water followed by 0.1% Tween-20 in TBS. Heat induced antigen retrieval was performed using a Digest-All™ 3 (Pepsin Solution; Invitrogen™, Life Technologies™). To block endogenous peroxidase activity, slides were treated with 0.5% H_2O_2 in methanol, for 20min at room temperature. To block non-specific binding, slides were exposed large volume Ultra V Block (Thermo Scientific™, Lab Vision™), for 30min at room temperature. Slides were subsequently incubated with mouse monoclonal antibody against pan Cytokeratin (Sigma-Aldrich®) at 1:200 in Large Volume UltraClean Diluent (Thermo Scientific™, Lab Vision™). After washing, sections were incubated with EnVision™ Detection System Peroxidase/DAB (Dako, Glostrup, Denmark) followed by hematoxilin staining. Invasion was evaluated under the microscope, in a blind fashion by three independent users, using a semi-quantitative approach taking into consideration the

behavior of the human cells (pan-Cytokeratin positive) in the CAM (scored 1 if invasive, and 0 if not invasive). The histological processing of CAM was performed in the Diagnostic Unit of IPATIMUP.

For statistical analysis of the angiogenic response was used GraphPad Prism® software. ANalysis Of VAriance (ANOVA) tests were used to calculate significance in an interval of 95% confidence level and values of $p<0.05$ were considered to be statistically significant. MKN28 cell are not naturally invasive or tumorigenic thus making it difficult to have clear histological slides. For this reason, statistical analysis of the invasion score was not performed given the small number of animals in each group.

CELL SURFACE ELISA.3.8

For cell surface Enzyme-Linked ImmunoSorbent Assay (ELISA), $5x10^4$ CHO cells (stably expressing WT or unfolded E757K mutant Ecad) were seeded in 24-well plate and, at 80% of confluence, were treated with 2% DMSO in culture medium for 24h. The unfolding was stimulated by removing the folding stimulus (incubation with normal medium for 4h). After treatments, cell surface density of Ecad was measured by primary antibody HECD-1, that recognized an extracellular epitope of Ecad, and HRP-conjugated secondary antibody in the presence of Amplex®Red (Molecular Probes®, Life Technologies™), a fluorescent HRP substrate, according as described by Kathryn W. Peters [72].

IMMUNOFLUORESCENCE.3.9

MKN28 cells were seeded on glass coverslips and, 48h after transfection, they were fixed and permeabilized with ice-cold methanol for 10min, washed and incubated with primary antibodies against (Table 3, annexes) diluted in PBS containing 5% Bovine Serum Albumin (BSA; Sigma-Aldrich®), for 1h at room temperature. Secondary antibodies (Table 4, annexes) were used as appropriate for 1h at room temperature in the dark. The coverslips were mounted on slides using Vectashield® mounting medium with 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, California, USA). Images were acquired using a confocal laser point-scanning microscope (Zeiss LSM 710).

RESULTS.04

4.1.1. Subcellular Distribution of DNAJB4 is Influenced by the Expression of a Mutant E-cadherin

To analyse the subcellular distribution of overexpressed DNAJB4, we used a subcellular fractionation kit in CHO cell lines stably expressing WT or unfolded E757K mutant Ecad. DNAJB4 protein was detected in all fractions isolated from both cell lines (Figure 8A). Interestingly, we observe a significant increase of the amount of DNAJB4 in the membranous fraction (that includes at least PM and ER markers) of cells that express mutant Ecad (Figure 8A). To clarify if this increase could be due to a fractionation to the PM, we isolated PM proteins by biotinylation assay, and found that DNAJB4 is present at the PM, but its overexpression is not influenced by the presence of WT or mutant Ecad (Figure 8B).

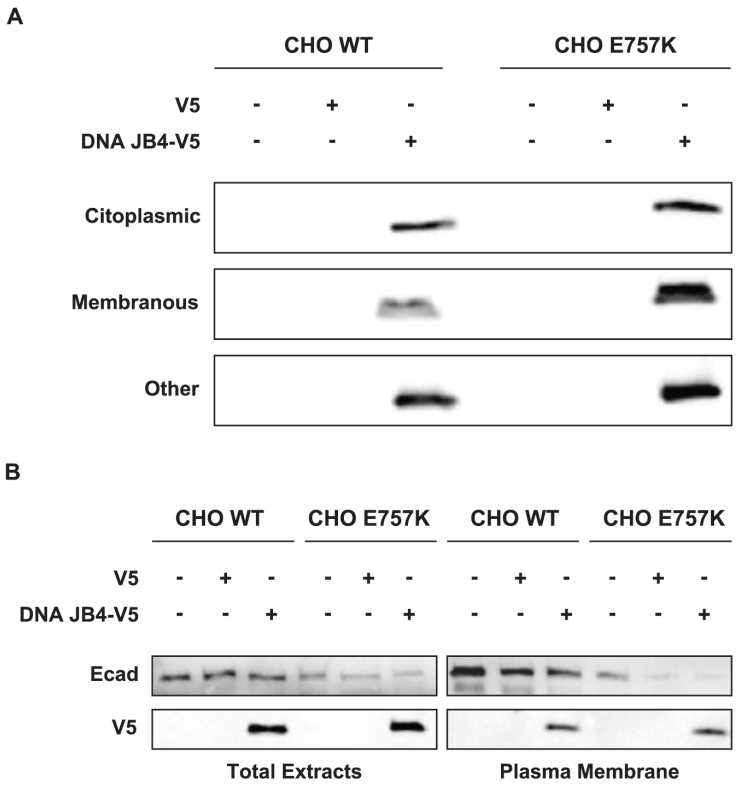
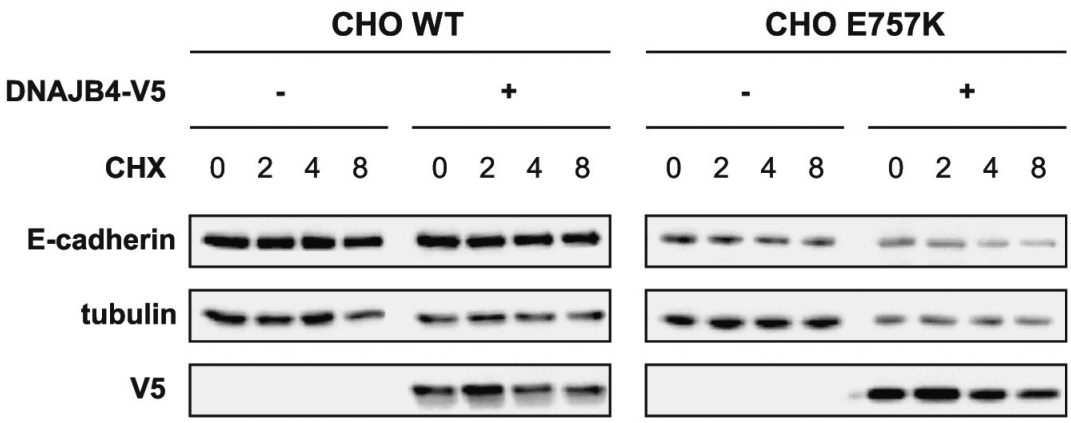


Figure 8. The amount of DNAJB4 is increased in the membranous fraction of the cells that express unfolded E757K mutant Ecad, but this increase is not due to PM recruitment. CHO cell lines stably expressing WT or E757K Ecad were transiently transfected with DNAJB4-V5 or with V5-tag (A) The fractions obtained with a subcellular fractionation kit are the following: citoplasmic, membranous and other (cytoskeleton, nucleous and cromatin). The fractions were analysed by WB, and DNAJB4 was detected with V5 antibody. (B) Biotinylation of cell-surface proteins was used to isolate PM proteins, and the presence of Ecad and DNAJB4 (with V5 antibody) was accessed by WB.

4.1.2. *DNAJB4 Determines the Half-life of Mutant E-cadherin*

Because the molecular chaperones are frequently determinants of the half-life of their substrates, we decided to determine if DNAJB4 regulates the half-life of Ecad. For this, we overexpressed DNAJB4 in CHO cells stably expressing WT and unfolded E757K mutant Ecad, and found that in the presence of DNAJB4 the half-life of the mutant is significantly reduced whereas the half-life of WT is not affected (Figure 9A and B).

A



B

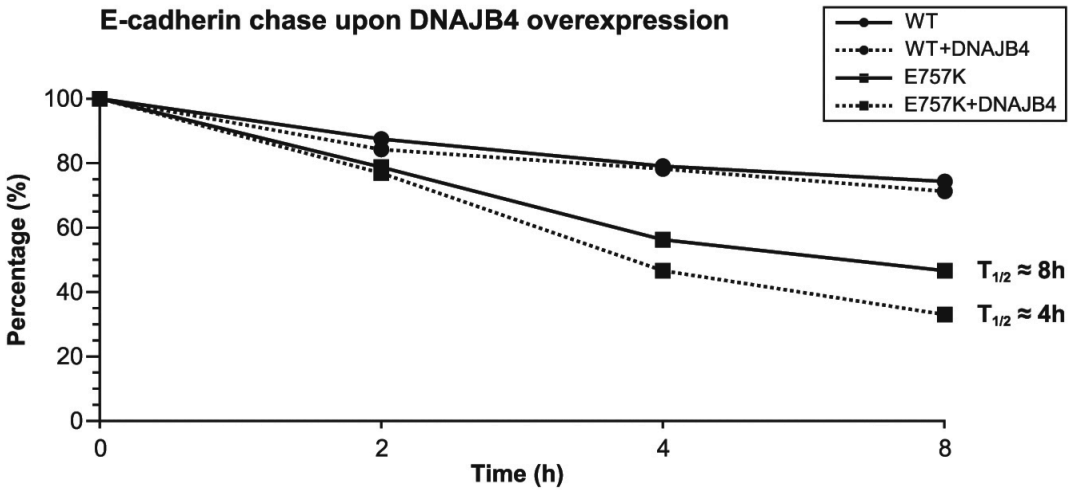


Figure 9. DNAJB4 reduces the half-life of unfolded E757K mutant Ecad. CHO cell lines stably expressing WT or E757K Ecad were transiently transfected with DNAJB4-V5 and, 24h after transfection, cells were incubated with the protein synthesis inhibitor Cicloheximide (CHX), for 0h, 2h, 4h and 8h. (A) Ecad expression was analysed by WB, transfection was confirmed with anti-V5 and α-tubulin served as a loading control for quantification purposes. (B) In each time point, Ecad expression was normalized to the control (0h, CHX). Circles represent WT cells and squares represent E757K mutant cells. Dashed lines represent cells upon overexpression of DNAJB4. The results are the average of three independent experiments.

4.1.3. DNAJB4 Mediates the Recognition of E-cadherin for Degradation in the Proteasome

As previously showed, unfolded E757K mutant Ecad is prematurely eliminated by the proteasome through the ERAD [42]. Therefore, we decided to test if DNAJB4 is involved in the recognition of Ecad for proteasome-dependent degradation. To achieve this, we performed an immunoprecipitation of WT and unfolded E757K mutant Ecad to analyse the interaction of DNAJB4 and Ecad under different conditions (Figure 10A). We observed that overexpressed DNAJB4 interacts with WT and mutant Ecad (Figure 10A, DNAJB4). This interaction is increased when we prevent degradation of Ecad by the proteasome (Figure 10A, DNAJB4+MG132) and is decreased when Ecad is stabilized by a chemical chaperone (Figure 10A, DNAJB4+DMSO). Moreover, the overexpression of DNAJB4 increases the amount of Ecad degraded in the proteasome (Figure 10B, DNAJB4+MG132), but not the amount of Ecad degraded in the lysosome (Figure 10B, DNAJB4+CQ). Interestingly, Ecad expression is highly stimulated by combination of overexpressed DNAJB4 and DMSO (Figure 10B, DNAJB4+DMSO).

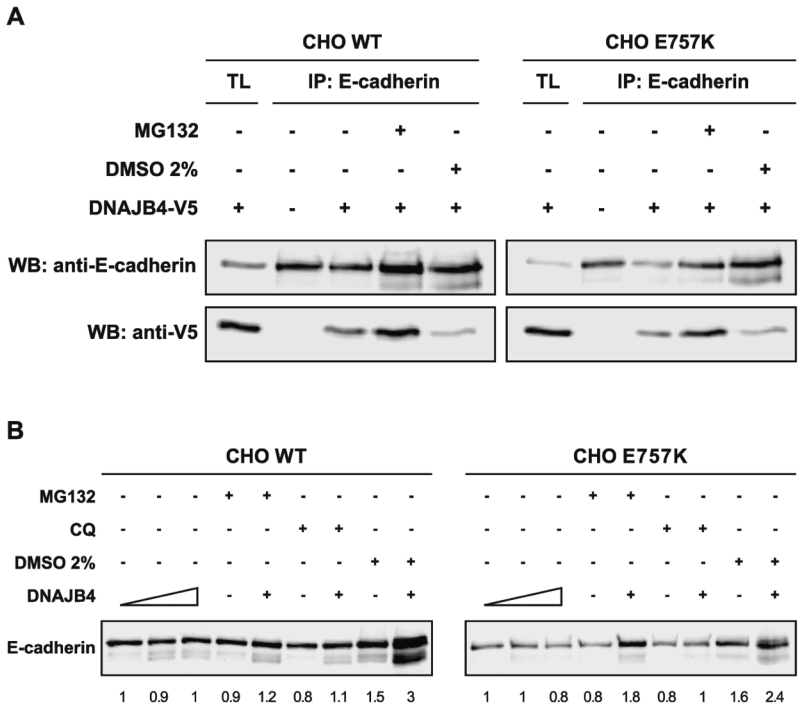


Figure 10. DNAJB4 mediates the recognition of Ecad for degradation in the proteasome, and potentiates the chemical effect of DMSO. CHO cell lines stably expressing WT or E757K Ecad were transiently transfected with DNAJB4-V5 and, 24h after transfection, cells were treated with MG132 (8h), 2% DMSO (24h) or CQ (6h), as indicated. (A) Immunoprecipitation was performed with anti-Ecad antibody, and WB against Ecad and V5 tag. (B) Total cell extracts were analysed by WB probed for Ecad, and the bands intensities were quantified. The result is representative of three biological replicas.

4.1.4. DNAJB4 Stimulates the Adhesion Role of WT E-cadherin in vitro

To evaluate the functional consequences of Ecad posttranslational regulation by DNAJB4, we performed aggregation and wound healing assays in different CHO cell lines transiently transfected with DNAJB4. We observed that the overexpression of DNAJB4 promotes the adhesion only in cells that expressed WT Ecad (Figure 11A). Addicionally, using the same models, DNAJB4 is not enough to reduce cell migration (Figure 11B).

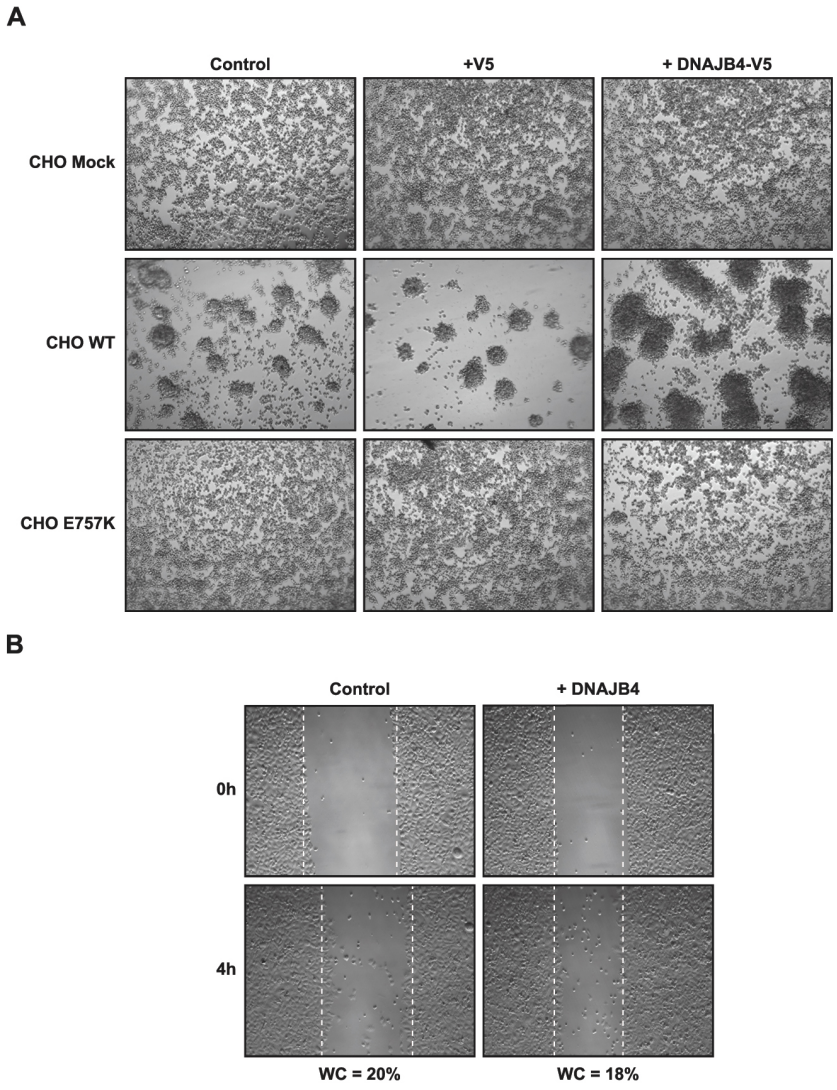


Figure 11. Posttranslational regulation of Ecad by DNAJB4 is sufficient to induce adhesion in cells expressing WT Ecad, but does not reduce cell migration. CHO cell lines stably expressing WT or E757K Ecad or an empty vector (Mock) were transiently transfected with DNAJB4-V5 or with V5-tag, as indicated. (A) Slow aggregation assay was performed and cells were photographed 48h after seeding. (B) Wound healing assay was executed with the same cells (WT), and images of wound closure (WC) were acquired every 2h, during 8h period. Representative images show for time points 0h and 4h. WC was quantified by measuring the distance between the wound edges in time, and the values represent percentage of closure. The experiment was repeated three times.

4.1.5. E-cadherin is Necessary for the Anti-angiogenic Effect of DNAJB4 in vivo

Angiogenesis is one of the hallmarks of the majority of cancers. Therefore, we decided to perform a CAM assay to analyse the angiogenic potential of DNAJB4 when expressed in MKN28 human GC cells. We established new GC cell models, by lentiviral transduction of shRNA against Ecad or the scramble control. Cells were selected by antibiotic resistance, and stable cells lines were obtained. We confirmed the silencing of Ecad by WB (Figure 12A).

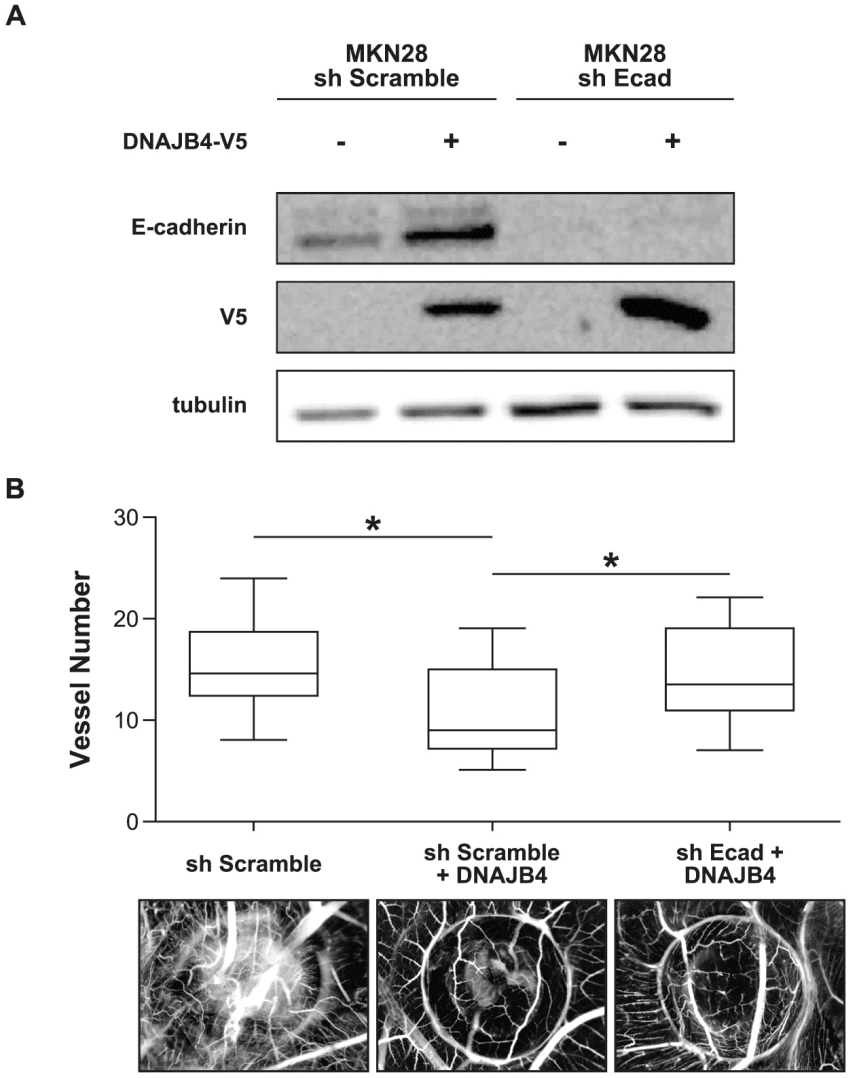


Figure 12. Ecad mediates the anti-angiogenic effect of DNAJB4 in a CAM assay of human GC cells. MKN28 cells stably transduced with sh Scramble or sh Ecad, were transiently transfected with DNAJB4-V5, as indicated. (A) 48h later, cells were processed for inoculation in the CAM by trypsin digestion and $5x10^4$ cells were lysed and analysed by WB. Ecad was detected with the BD antibody against the cytoplasmic tail, and DNAJB4 was detected with anti-V5 antibody. α-tubulin staining was used as a loading control. (B) CAM inoculated with DNAJB4 overexpressing cells (n=12) has significantly ($p<0.05$) fewer new vessels than the same cells without transfection (n=12) or with stable Ecad silencing (n=14). Representative images of the CAM show abnormal vascular structure and numerous neovessels growing towards the inoculation area (delimited by the ring).

These cell lines, with stable silencing of Ecad (sh Ecad) or with stable expression of sh control (sh Scramble), were both transiently transfected with DNAJB4, as confirmed by WB (Figure 12A). The cells were inoculated over the CAM and processed for subsequent analysis. We found that overexpression of DNAJB4 decreases the number of vessels formed, as compared to the control cells (Figure 12B). Interestingly, in the absence of Ecad expression, the anti-angiogenic phenotype of DNAJB4 is reversed (Figure 12B).

4.1.6. E-cadherin is Necessary for the Anti-invasive Effect of DNAJB4 in vivo

Because invasion is also one of the hallmarks of carcinogenesis, we used the same cells. CAM assay to evaluated the invasive potential of DNAJB4 by immunohistochemistry. For this, we used the cell lines described in the previous experience and verified that DNAJB4 is anti-invasive, but this tumor suppressor phenotype is lost in Ecad silencing conditions (Figure 13A and B).

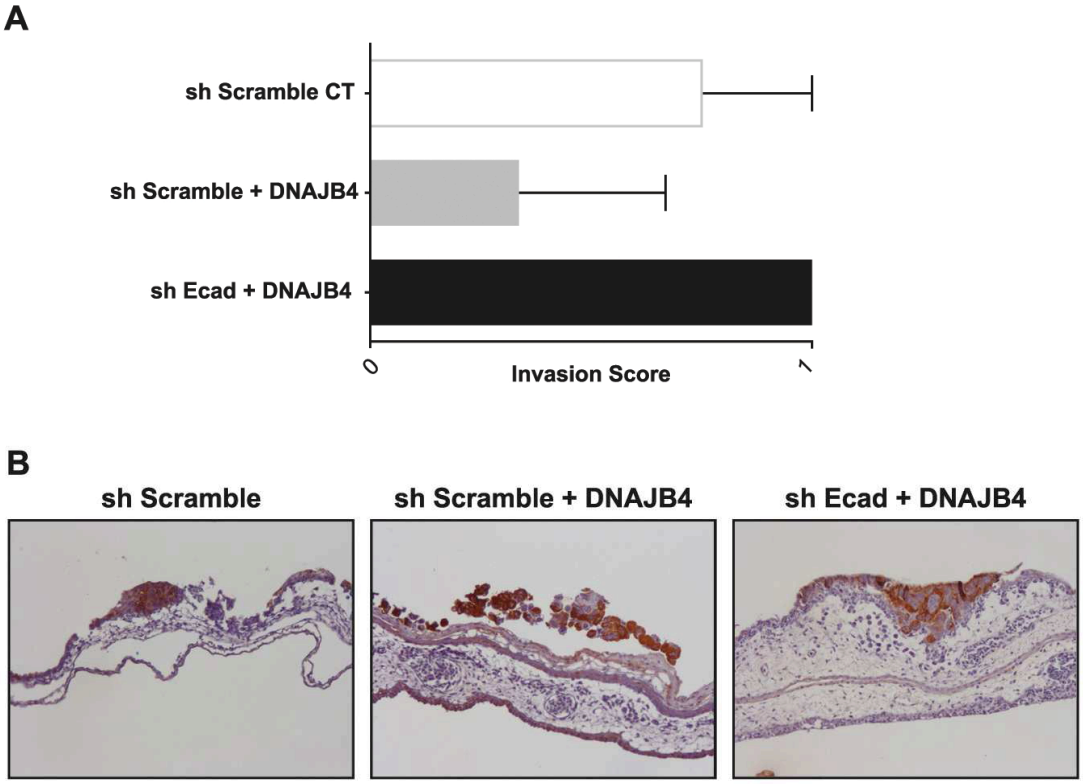


Figure 13. Ecad mediates the anti-invasive effect of DNAJB4 in a CAM assay of human GC cells. MKN28 cells stably transduced with short-hairpin RNA scramble (sh Scr) or against Ecad (sh Ecad), were transiently transfected with DNAJB4-V5, as indicated. A human-specific cytoqueratin staining allowed the distinction of inoculated cells and the evaluation of cell invasion over the CAM (A) cells were scored as Invasive (1) or Non-invasive (0). At least three independent experiments were used for the graphic representation. (B) Representative images of the invasion of the same cells over the CAM.

4.2.1. *Hsc70 Regulates the Endocytosis of Unfolded E-cadherin*

As previously mentioned, PPQC can be involved in the regulation of unfolded proteins at the PM. The molecular chaperone Hsc70 is one of the partners of this mechanism that participates in the recognition of these proteins [66]. So, we decided to test if Hsc70 plays a role in the regulation of Ecad endocytosis in the presence of an extracellular stimulus that leads to the unfolding of this protein. Calcium depletion is described to induce denaturation of the Ecad extracellular domain, resulting in its internalization and consequent degradation in the lysosome [73]. We used MKN28 cells under conditions of calcium chelation and Hsc70 silencing and found that, upon Hsc70 silencing, Ecad is reduced or destabilized (condition with calcium) and its internalization/degradation is compromised, as observed from the lack of Ecad reduction upon calcium depletion, compared to the conditions with normal Hsc70 levels (Figure 14A).

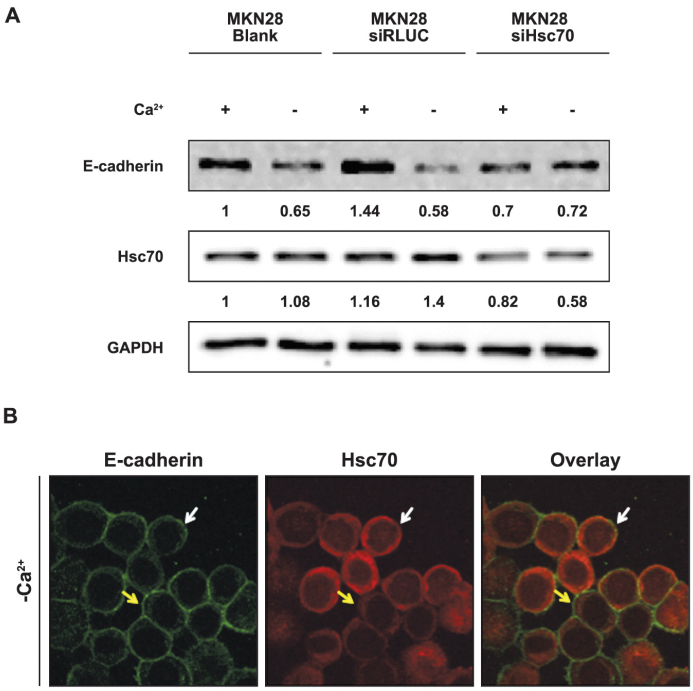


Figure 14. Hsc70 regulates internalization of Ecad from the PM upon extracellular calcium depletion. MKN28 cells were transiently transfected with siRLUC (non-target) or siHsc70 and, 48h after transfection, cells were incubated with medium without calcium supplemented with EDTA (2mM, 10 min). (A) Total cell lysates were analised by WB, probed for Ecad, Hsc70 and GAPDH, served as a loading control for quantification purposes. (B) Ecad and Hsc70 were detected by IF with specific antibodies, coupled to Alexa 488 and Alexa 568 respectively. White arrows point to Hsc70-high cells that exhibit typical Ecad loss at the PM, and yellow arrows point to Hsc70-low cells, that retain Ecad in the PM.

To evaluate if Hsc70 regulates the internalization by endocytosis of unfolded WT Ecad at the PM, we performed an immunofluorescence (IF) against Ecad and Hsc70, in the same conditions of the previous experience. We observe a loss of Ecad at the PM in cells that exhibit high levels of Hsc70, in contrast to the cells in which the expression of Hsc70 is decreased (Figure 14B).

4.2.2. Unfolded E-cadherin Leads to a Recruitment of Hsc70 and CHIP to the Plasma Membrane

To further investigate the putative role of PPQC in the regulation of unfolded Ecad at the PM, we decided to use the intrinsically unfolded E757K mutant Ecad as an additional experimental model. To achieve this, we needed to rescue this unfolded Ecad from ERAD and recover its expression at the cell surface.

Simões-Correia *et al.* showed that chemical chaperone DMSO promotes the folding of E757K Ecad and restored this mutant to the PM. Subsequently, removal of the folding stimulus leads to unfold and rapid internalization of the mutant [42]. To confirm this, we optimized a cell surface ELISA assay described by Kathryn W. Peters [72], using CHO cells stably expressing WT (as a control) and unfolded E757K mutant Ecad. We confirmed that under basal conditions, the unfolded E757K mutant Ecad is less expressed at the PM than WT Ecad (Figure 15). Furthermore, the expression of mutant Ecad at the cell surface increases upon incubation with DMSO and returns to lower expression levels after removal of the folding stimulus (Figure 15). These results were consistent with what has been shown by Simões-Correia *et al.* [42].

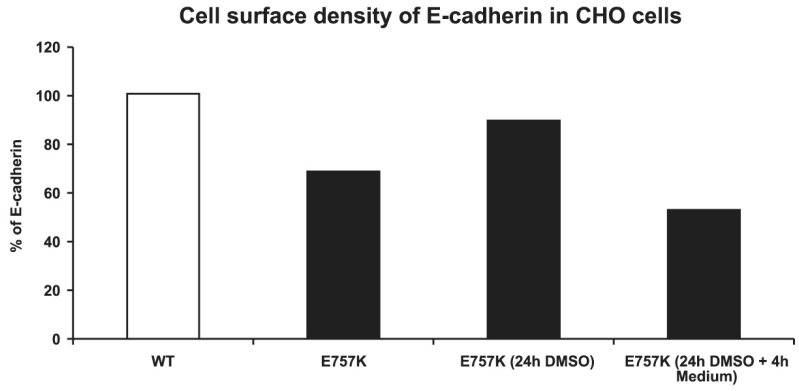


Figure 15. DMSO induces a reversible rescue of unfolded Ecad to the PM. CHO cells stably expressing E757K mutant Ecad were incubated for 24h with 2% DMSO followed of 4h with normal medium or incubated for 24h with or without 2% DMSO. CHO cells stably expressing WT Ecad were used as a control for Ecad expression at the PM. Density of Ecad at the PM was measured by cell surface ELISA.

In addition to Hsc70, CHIP E3 Ub-ligase also belongs to PPQC machinery, playing a role in the ubiquitination of unfolded proteins at the PM [66]. Accordingly, we decided to analyse if both molecular partners are involved in the regulation of unfolded Ecad (resulting from either an extracellular stimuli or genetic mutation) at the cell surface. For this, we isolated PM proteins by biotinylation assay and verified that the expression of unfolded mutant Ecad leads to a recruitment of Hsc70 and an increase of CHIP at the PM, comparing to WT Ecad (Figure 16, Control). In contrast, we found that when mutant Ecad is stabilized using DMSO, Hsc70 is not recruited and the amount of CHIP is decreased at the cell surface (Figure 16, DMSO). We also observe an increase of CHIP at the PM when we induced destabilization of mutant Ecad after treatment with the folding stimulus (Figure 16, DMSO+Medium). Moreover, when we induced the unfolding of WT Ecad and inhibited lysosomal degradation (CQ) and endocytosis (sucrose) we observe a recruitment of Hsc70 and an increase of CHIP levels at the cell surface (Figure 16, EDTA+CQ and EDTA+Sucrose). Interestingly, when we induce only the destabilization of Ecad we found that Hsc70 is not recruited and amount of CHIP is reduced at the PM (Figure 16, EDTA). In the other hand, we observe a slight recruitment of Hsc70 and an increased amount of CHIP at the cell surface when we blocked the lysosome (Figure 16, CQ).

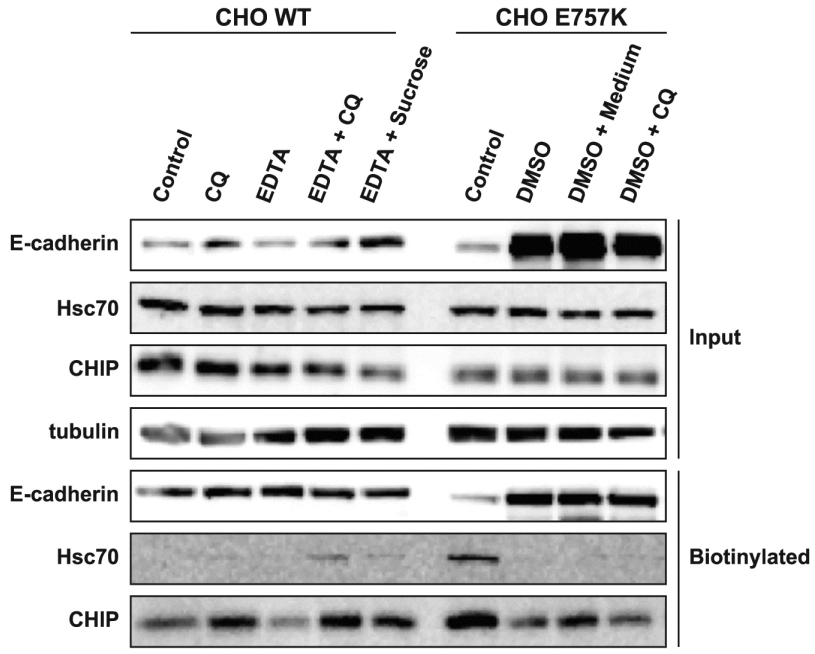


Figure 16. Accumulation of unfolded Ecad leads to a recruitment of Hsc70 and CHIP to the PM. CHO cells that express WT Ecad were incubated with/without CQ (100μM, 1h), or with/without EDTA (2mM, 30 min), or with/without EDTA (2mM, 30 min) and CQ (100μM, 1h), or with/without EDTA (2mM, 30 min) and sucrose (0,4M, 15 min). CHO cells expressing E757K Ecad were incubated for 24h with 2% DMSO followed of 1h with CQ (100μM), or 24h with 2% DMSO followed of 4h with normal medium, or 24h with/without 2% DMSO. Total cell extracts were analysed by WB and biotinylation of cell-surface proteins was used to separate PM proteins, and WB accessed the levels of Ecad, Hsc70 and CHIP.

4.2.3. *Silencing of CHIP Potentiates the Adhesion Role of WT E-cadherin*

To evaluate the functional consequences Ecad posttranslational regulation by Hsc70 and CHIP, we performed an aggregation assay in CHO cells, stably transduced with empty vector (Mock), WT or E757K mutant Ecad and transiently transfected with siHsc70 or siCHIP. Because silencing of Hsc70 was not effective (Figure 17A) we present only the effect of silencing of CHIP in cell adhesion. Since it was not possible to optimize this assay in order to include the stimuli used in the previous experiment, we decided to focus on analyzing only the effect of CHIP depletion under basal conditions. We observed that silencing of CHIP promotes adhesion only when WT Ecad is expressed (Figure 17B), but has not effect in conditions where there is no Ecad expression, or with if a mutant Ecad is expressed (Figure 17B).

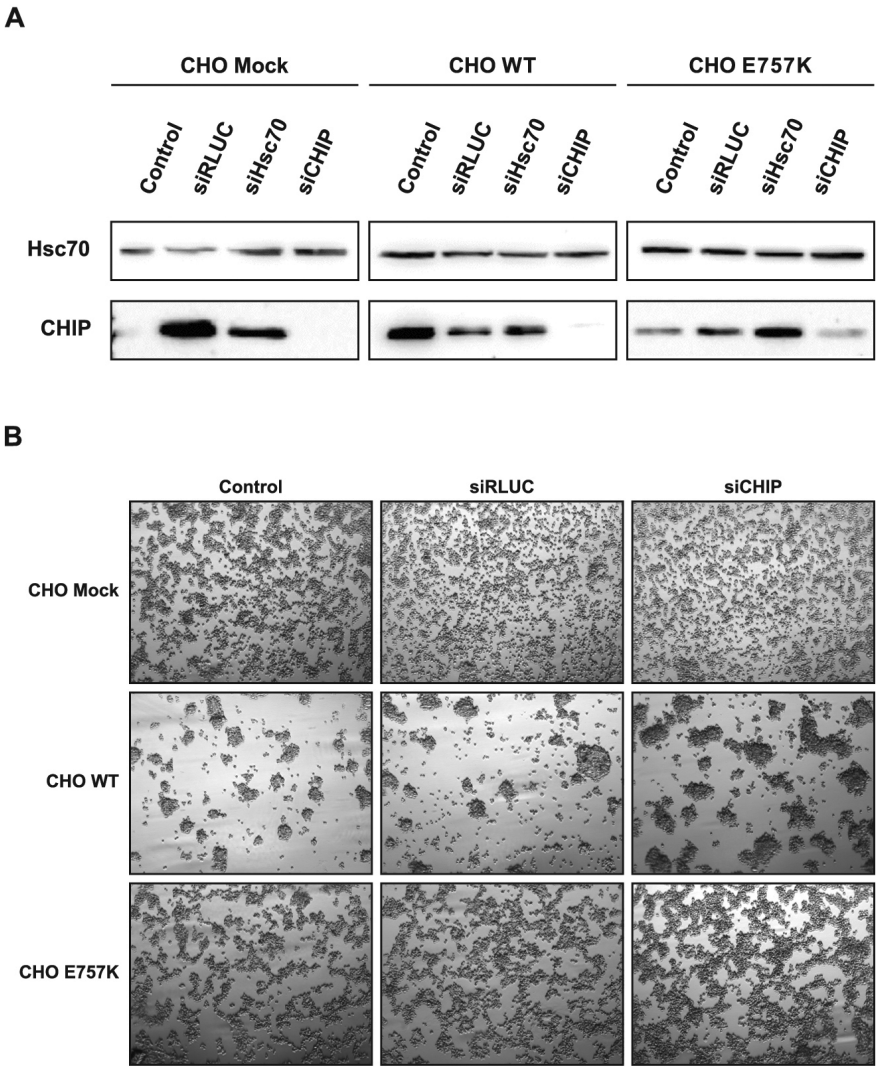


Figure 17. CHIP regulates the adhesive properties of WT Ecad. CHO cell lines stably expressing WT or E757K Ecad or an empty vector (Mock) were transiently transfected with siRLUC (non target), siHSC70 or siCHIP, as indicated. Slow aggregation assay was performed and cells were photographed 48h after seeding.

DISCUSSION.05

Ecad is a calcium dependent cell adhesion molecule. It is the major component of AJ and is essential for the establishment and maintenance of polarization and differentiation of embryonic and adult epithelial tissues [7,42]. Mutations in Ecad gene are often associated with cancer progression and poor patient prognosis [7,42,30]. An example that illustrates the carcinogenic potential of these mutations is HDGC. A small proportion (28%) of germline mutation identified in HDGC are missense that gives rise to single AA substitution, resulting in a codon that codes for a different AA [74]. HDGC-associated *CDH1* germline missense mutations often lead to folding defects of Ecad that are surveyed by quality control mechanisms [42,45]. These mechanisms play an important role in the regulation of Ecad expression and function.

Simões-Correia *et al*. demonstrated that mutants of Ecad associated to the HDGC are subjected to the ER quality control that leads a loss of Ecad surface expression. However, a small fraction of these mutants escape ERAD and are trafficked to the PM without achieving proper folding [42].

Recent studies suggest that unfolded proteins at the cell surface that either result from genetic mutations or denaturing extracellular stimuli can be regulated by PM quality control mechanisms [66-67].

In this work, we explored the role of the molecular chaperone DNAJB4 in the regulation of Ecad associated to the ER and investigated if unfolded Ecad could be regulated by PPQC mechanisms at the PM.

5.1. Endoplasmic Reticulum Quality Control

ER quality control plays a major role in Ecad regulation [42]. However, the molecular chaperones involved in the specific degradation of Ecad were not known.

From a genetic screen for Ecad interactors, in the *Drosophila* fly, we identified DnaJ-1 (human homolog DNAJB4) as a partner that may be related with folding, stability and/or protein degradation (data not shown).

DNAJB4 is an Hsp40-like molecular chaperone that was previously described to act as a TSG in NSCLC. In this context, DNAJB4 promotes the indirect induction of Ecad expression at the transcriptional level, by downregulation of its transcriptional repressor

Slug [65,75]. Interestingly, it has also been shown that in lung cancer cell models, curcumin induces transcription of DNAJB4 and increases Ecad expression [76]. Because curcumin is described to act as a chemical chaperone [77] and is an inducer of the heat shock response [78], we hypothesized that the regulation of Ecad by DNAJB4 expression could also happen at the posttranslational level. To prove our hypothesis, we used cadherin-null CHO cell lines stably transduced with WT or unfolded E757K mutant Ecad lacking the proximal promoter region subjected to regulation by the transcriptional repressor Slug.

After subcellular protein fractionation, DNAJB4 was detected in citoplasmic, membranous and other (cytoskeleton, nucleous and cromatin) fractions, of CHO cells expressing WT or unfolded E757K mutant Ecad (Figure 8A). Interestingly, the amount of DNAJB4 is increased in the membranous fraction of cells that express unfolded mutant Ecad (Figure 8A). This fraction includes at least PM and ER proteins (as inferred by the presence of specific markers). However, this increase is not observed at the PM, as inferred from the biotynilation assay, used to detect cell surface proteins (Figure 8B), suggesting that the enrichment of DNAJB4 in the membranous fraction is associated to other membranes (e.g. ER) and not to increased PM recruitment. Previously other authors raised the hypothesis that DNAJ proteins are associated with ER [58].

Using cicloheximide to inhibit protein synthesis, we show that DNAJB4 overexpression reduces the half-life on unfolded E757K mutant Ecad (Figure 9). This result supports the hypothesis that DNAJB4 regulates Ecad at the posttranslational level, inducing unfolded Ecad degradation.

We also demonstrate that DNAJB4 interacts with Ecad and this interaction is increased under proteasome inhibition (Figure 10A, DNAJB4+MG132) suggesting that DNAJB4 preferentially interacts with the proteasomal degradation-prone fraction of Ecad. In the opposite direction, when Ecad is stabilized by chemical chaperone treatment, the interaction between Ecad and DNAJB4 is decreased (Figure 10A, DNAJB4+DMSO). This confirms that DNAJB4 preferentially binds unfolded Ecad. Moreover, the overexpression of DNAJB4 increases the fraction of Ecad degraded in the proteasome (Figure 10B, DNAJB4+MG132) suggesting that it mediates the degradation of unfolded Ecad by the proteasome. Interestingly, DNAJB4 indirectly potentiates the DMSO effect of Ecad stabilization (Figure 10B, DNAJB4+DMSO). This is consistent with other authors, where it is described that DMSO promotes the multiple functions of DNAJB4 [75].

After we have partially elucidated the mechanism whereby DNAJB4 influences Ecad stability at the posttranslational level, we investigated the functional consequences of this regulation. We observe that overexpression of DNAJB4 is not sufficient to increase cell

adhesion in the absence of Ecad or in the presence of the non-functional unfolded E757K mutant Ecad, but induce increased cell aggregation in the presence of WT Ecad (Figure 11A) suggesting that its pro-adhesive role is cadherin-dependent. This was expected, because it increases the amount of WT Ecad in the PM (data not shown). To further analyse the role of DNAJB4 in cell migration, we used the wound healing assay. The results show that, in conditions of WT Ecad without transcriptional regulation, DNAJB4 is not enough to reduce cell migration (Figure 11B), indicating that Ecad dominantes over DNAJB4 for the regulation of cell migration.

Analysis of the neovascularizing potential of the human cells over the CAM shows that DNAJB4 is anti-angiogenic, and that this tumor suppressor feature is also Ecad-dependent (Figure 12). Labeling of these cells inoculated in the CAM revealed that DNAJB4 stimulates the anti-invasive function of WT Ecad, but this invasion-suppressor potential is lost if Ecad is not expressed (Figure 13), suggesting that in the GC model Ecad is the dominant anti-invasive molecule.

5.2. Plasma Membrane Quality Control

PPQC is a recently described specialized pathway for the regulation of unfolded proteins at the PM. Molecular components of this pathway include chaperones, co-chaperones and ubiquitinating enzymes [66]. In this work we sought to test the hypothesis that unfolded Ecad at the cell surface can be regulated by PPQC components such as Hsc70 and CHIP.

To clarify this hypothesis, we used two different experimental models: unfolded WT Ecad at the PM, resulting from an extracellular stimuli (calcium depletion), and an intrinsically unfolded mutant Ecad, rescued to the cell surface by treatment with the chemical chaperone DMSO.

Under conditions of extracellular calcium depletion, we observed a decrease in the total levels of Ecad (Figure 14A). However, in cells with stable silencing of Hsc70, the amount of Ecad is not affected under the same conditions (Figure 14A). Additionally, after calcium chelation there was a loss of Ecad at the PM in cells that exhibit high levels of Hsc70, in contrast to the cells in which the expression of Hsc70 is decreased (Figure 14B), indicating that Hsc70 regulates the endocytosis of unfolded Ecad. Some studies demonstrated that extracellular calcium depletion is one of main factors that significantly increase the process of Ecad clathrin-mediated endocytosis [73], namely because it is recognized as a denaturing stimulus for the extracellular domain of Ecad denaturation.

These results suggest that Hsc70 is likely to be involved in the recognition of unfolded Ecad at the PM for internalization and subsequent degradation. This is in accordance with the theoretical model of PPQC, where Hsc70 is predicted to play a role in the recognition of the unfolded protein substrates. However, Hsc70 also plays multiple roles in the endocytic pathway. First Hsc70 is required for budding of Clathrin-Coated Vesicles (CCV). It then participates in uncoating of CCV to allow the fusion of these vesicles with the early endosome. Finally, Hsc70 may be involved in the rebinding of clathrin to the PM to form new CCV [79, 80]. Given that Ecad undergoes clathrin-mediated endocytosis, our results might also suggest that the Hsc70 effect in Ecad endocytosis is dependent on its chaperoning role over clathrin, and not directly on unfolded Ecad. Therefore, it is not clear if Hsc70 is indeed playing a role in the recognition of unfolded Ecad at the PM, or generally influencing clathrin-dependent endocytosis.

Through biotinylation of cell-surface proteins we show that the expression of unfolded mutant Ecad leads to a recruitment of Hsc70 and an increase of CHIP at the PM, comparing to the conditions with WT Ecad expression (Figure 16, Control). In contrast, when mutant Ecad is stabilized using DMSO, Hsc70 is not recruited and the amount of CHIP is decreased at the cell surface (Figure 16, DMSO). In the other hand, when we remove the stabilizing stimulus for mutant Ecad the levels of CHIP increase (Figure 16, DMSO+Medium). Moreover, when we induced the unfolding of WT Ecad and inhibited endocytosis/lysosomal degradation we verify a recruitment of Hsc70 and an increase of CHIP levels at the cell surface (Figure 16, EDTA+Sucrose and EDTA+CQ). Taken together, these results show that Hsc70 and CHIP are dynamically recruited to the PM in response to Ecad unfolding and suggest their participation in its regulation at the PM.

Interestingly, in cells expressing WT Ecad, calcium depletion *per se* did not result in the recruitment of Hsc70 and reduced the amount of CHIP at the PM (Figure 16, EDTA). This may be a consequence of the rapid internalization of membrane proteins upon the unfolding stimulus. As the affected proteins enter the endocytic pathway, only the properly folded membrane proteins (wich are not targeted by the PPQC machinery) are left in the membrane, thus decreasing the levels of Hsc70 and CHIP at this site. In the other hand, we observe a slight recruitment of Hsc70 and an increased amount of CHIP at the cell surface when we blocked the lysosomal degradation (Figure 16, EDTA+CQ) or endocytosis (Figure 16, EDTA+Sucrose). These results suggest that Hsc70 and CHIP are recruited when we accumulate unfolded Ecad at the PM.

Because CHIP could regulate Ecad stability at the posttranslational level, we investigated the functional consequences of this regulation, and observed that silencing of CHIP potentiates de adhesive properties of WT Ecad (Figure 17B). Although cells were not exposed to an unfolding stimulus, this result is in line with the hypothesis that CHIP does

regulate Ecad at the PM and is likely to reflect the role of CHIP in targeting damaged WT Ecad for degradation.

CONCLUDING REMARKS.06

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We conclude that DNAJB4 is a molecular chaperone of Ecad. This newly described interaction partner is able to distinguish between properly folded Ecad and its unfolded counterpart (e.g. resulting from hereditary/sporadic mutation, or unfolding due to a stress insult during cancer progression), directing Ecad towards the PM (WT Ecad) or to the proteasome for degradation (unfolded Ecad).

The posttranslational regulation of Ecad by DNAJB4 has functional consequences at the level of cell adhesion, but not cell migration. Our *in vivo* data suggests that in GC DNAJB4 has an anti-angiogenic and anti-invasive effect, if Ecad expression is retained.

Recent work reported that DNAJB4 acts as a tumour suppressor in NSCLC. However, in GC, Ecad is frequently lost or mutated, meaning that the DNAJB4 expression might promote tumour progression. We believe that DNAJB4 acts as a conditional tumour suppressor in carcinomas, where its function depends of the presence of Ecad.

In addition, we suggest that PPQC is involved in the regulation of Ecad at the PM, and that this level of regulation is especially relevant when Ecad is unfolded due to mutation or to an external denaturing stimulus.

Indeed, Hsc70 is a regulator of Ecad endocytosis and CHIP regulates the adhesive properties of Ecad. However, further experiments are required to clarify the roles of these two key players of PPQC in the regulation of Ecad expression at the PM.

In summary, we conclude that PQC plays a major role in the regulation of Ecad levels and function. Understanding this mechanism of regulation will help us to design new therapeutic strategies for HDGC and other cancers associated to Ecad mutations.

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ANNEXES

Table 1. Identification of plasmids and their specifications.

Insert	Vector backbone	Number	Reference
E-cadherin shRNA	pLKO.1 puro	18801	Addgene
Scramble shRNA	pLKO.1	1864	Addgene
DNAJB4	pcDNA5/FRT/TO	19472	Addgene
V5 DNAJB4	pcDNA5/FRT/TO	19526	Addgene
V5 ()	pcDNA5/FRT/TO	19445	Addgene

Table 2. Identification of siRNAs and their specifications.

Gene Name	Specie	Catalog Number	Reference
HSPA8	Human (ENO1 3312)	EHU115141	Sigma-Aldrich®
STUB1	Human (STUB1 10273)	EHU106761	Sigma-Aldrich®
RLUC	Human (MED23 9439)	EHURLUC	Sigma-Aldrich®

Table 3. Identification of primary antibodies and their general specifications for WB and IF.

Antibody	Source	Concentration (WB)	Concentration (IF)	Reference
E-cadherin (HECD-1)	Mouse	1:2500	—	Novex®
E-cadherin	Rabbit	—	1:200	Cell Signaling Technology®
V5	Mouse	1:1000	—	Novex®
α-tubulin	Mouse	1:1000	—	Sigma-Aldrich®
Hsc70	Rabbit	1:1000	—	Pierce Biotechnology
Hsc70	Rat	1:1000	1:100	Enzo®Life Sciences
CHIP (STUB1)	Goat	1:500	—	Abcam®
CHIP	Rabbit	1:1000	—	Cell Signaling Technology®
GAPDH	Goat	1:5000	—	Sicgen
Calnexin	Goat	1:2000	—	Sicgen

Table 4. Identification of secondary antibodies and their general specifications for WB and IF.

Antibody	Source	Conjugate Type	Concentration (WB)	Concentration (IF)	Reference
Mouse	Goat	HRP	1:5000	—	Bio-Rad
Rabbit	Goat	HRP	1:5000	—	Bio-Rad
Goat	Rabbit	HRP	1:5000	—	Novex®
Rat	Goat	HRP	1:5000	—	Novex®
Mouse	Goat	Alexa Fluor® 568	—	1:250	Molecular Probes®
Rabbit	Goat	Alexa Fluor® 488	—	1:250	Molecular Probes®
Rat	Rabbit	Alexa Fluor® 568	—	1:250	Molecular Probes®